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(71) Applicant: TOSOH CORPORATION
4560, Oaza-tonda Shin-nanyo-shi
Yamaguchi-ken 746(JP)

Applicant: RIKEN INSTITUTE OF PHYSICAL AND CHEMICAL RESEARCH
2-1, Hirosawa
Wako-shi, Saitama-ken, 351-01(JP)

(72) Inventor: Ogawa, Kazuo
1-7-1 Aoba-cho, Higashi
Murayama-shi, Tokyo 189(JP)
Inventor: Nishi, Akihiro
2398, Kawaraguchi
Ebina-shi, Kanagawa-ken 243-04(JP)
Inventor: Koyama, Kenji
1775, Okada
Atsugi-shi, Kanagawa-ken 243(JP)
Inventor: Matsuno, Takanori
2398, Kawaraguchi
Ebina-shi, Kanagawa-ken 243-04(JP)

(74) Representative: Kearney, Kevin David Nicholas et al
KILBURN & STRODE 30 John Street
London, WC1N 2DD(GB)

(54) Method for purifying phage DNA.

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EUROPEAN SEARCH REPORT

Application Number

EP 90 31 3173

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	EP-A-240191 (SEIKO INSTRUMENTS INC.) * column 2, line 35 - column 3, line 49 * ---	1-4	C12N15/10 C12P19/34
A	WO-A-8901035 (EUROPAISCHES LABORATORIUM FUR MOLEKULARBIOLOGIE_(EMBL)) * page 3, line 12 - page 4, line 30 * ---	1	
P,A	EP-A-376080 (TALENT SRL) * column 2, line 35 - column 4, line 29 * -----	1-4	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	28 FEBRUARY 1991	ANDRES S.M.

CATEGORY OF CITED DOCUMENTS

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PRODUCT APPLICATION FOCUS

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Use of Ultrafiltration Microconcentrators in the Concentration and Desalting of DNA

F. Marashi[1,2], G. Stein[1], J. Stein[1], and C. Schubert[3]
[1]Univ. of Florida College of Medicine, [2]Phillips Petroleum Co. and [3]Amicon Corporation

Concentrating dilute DNA solutions is a key step for numerous preparative and analytical procedures. For example, standard plasmid preparations involving cesium chloride equilibrium centrifugation and gel filtration yield DNA in large volumes that require concentrating prior to precipitation. Another situation which necessitates concentrating DNA is the purification of restriction fragments from gels, where high concentrations facilitate efficient and complete precipitation. Current techniques that are available for concentrating nucleic acids include: 1) repeated extractions with n-butanol, 2) adsorption to ion exchange resin followed by high salt elution, and 3) lyophilization. The first method has the disadvantage that the n-butanol concentrates all solutes, including salt which tends to coprecipitate with DNA upon addition of ethanol. The ion exchange method, aside from requiring buffers of various ionic strengths, yields DNA preparations in a high salt solution. Lyophilization also increases the concentration of buffer components which can result in degradation of nucleic acids.

Ultrafiltration provides an effective alternative for rapidly concentrating as well as desalting plasmid DNAs and plasmid DNA restriction fragments. With Amicon's Centricon™ Disposable Microconcentration System (Figure 1), desalting is achieved by ultrafiltration of the solution through a membrane, resulting in the removal of ionic buffer components. The concentrated polynucleotides are retained by the membrane, as shown in Figure 1. Driving force for filtration is provided by fixed-angle centrifugation at 1000 to 5000 × g. This system incorporates low-adsorptive filter membranes for minimal non-specific adsorption of DNA fragments (typically less than 5%). They retain fragments as small as 75 base pairs. We have tested the Amicon Centricon ultrafiltration system for concentrating solutions of plasmid DNA supercoils, restriction endonuclease fragments of a plasmid DNA ranging in size

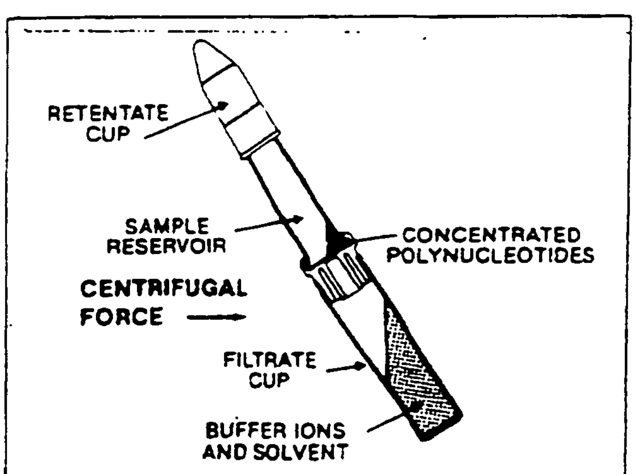

Figure 1. Schematic of the Centricon Microconcentrator.

1 2

Figure 2. Ethidium bromide staining of linearized pBR322 concentrated by Centricon-30 microconcentrator. One hundred nanograms of linearized plasmid DNA was diluted to 1.0 ml with TE, and was subsequently concentrated to 40 μl using the Centricon-30 microconcentrator. Lane 1 — 50 ng of linearized plasmid DNA; lane 2 — 20 μl of the sample concentrated using the Centricon-30 microconcentrator. The samples were electrophoresed in a 2.5% agarose gel which was stained in a solution of 1 μg/ml ethidium bromide in distilled water.

ized pBR322 plasmid DNA. As shown in Figure 2, >99% recovery of the ligated plasmid DNA was obtained. No evidence of degradation can be observed. Recovery was quantitated by absorption at A_{260} of samples prior to electrophoretic fractionation and by microdensitometry of photographs of ethidium bromide-stained gels.

The biological activity of plasmid DNA that had been concentrated using the Amicon Centricon-30 microconcentrator unit was evaluated. pBR322 plasmid DNA was linearized by cleavage with *Eco*RI, concentrated in the Centricon-30 microconcentrator and assayed for transformation efficiency following recircularization and ligation. In comparison to a plasmid that was similarly restricted and religated but concentrated by ethanol precipitation, the preparation concentrated in the Centricon-30 microconcentrator unit was equally effective for transfection of *E. coli* HP101 (Table 1).

The effectiveness of the Centricon-10 and Centricon-30 microconcentrator units was compared. Figure 3 shows that both Centricon-10 and -30 filters may be used in concentrating double-stranded, ^{32}P-labeled DNA fragments as small as 75 base pairs with very good recovery (>99% at solute concentrations of 10 μg-100 μg/ml). Analyzing the filters by liquid scintillation counting demonstrated that the filters do not adsorb significant amounts (less than 5%) of double-stranded DNA under the conditions used. The same results were obtained for radiolabeled single-stranded DNA fragments of approximately 200 nucleotides.

While both the recovery and biological activity are similar when DNAs are concentrated using the Centricon microconcentrator units or conventional techniques, the efficiency and immediate ability to further utilize the nucleic acids concentrated with the Centricon unit without subsequent desalting steps is a significant advantage. The self-contained nature of the Centricon-10 and -30 microconcentrators make them extremely convenient to use. Although the maximal volume that can be concentrated in a single centrifugation is 2.5 ml, larger samples can be concentrated by repeated centrifugation runs.

In addition to concentrating and desalting DNA preparations, Centricon is effective for recovery and purification of proteins, enzymes, antibodies and other macromolecules from biological samples such as serum, cell culture extracts and lysates. □

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For additional information or reprints contact C. Schubert, Amicon Corp., 17 Cherry Hill Dr., Danvers, MA 01923.

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(54) Method for purifying phage DNA.

(57) Disclosed is a method for purifying phage DNA which can be carried out without centrifugation steps and without use of toxic reagents. In the method of the invention, a mixture containing microorganism cells and phage is filtered through a membrane filter so as to remove said microorganism cells. Then phage proteins are decomposed and denatured. Thereafter, the resultant is subjected to ultrafiltration so as to remove impurities.

EP 0 431 905 A1

BACKGROUND OF THE INVENTION

I. Field of the Invention

The present invention relates to a method for purifying phage DNA. The method of the present invention may be used in the field of genetic engineering.

II. Description of the Related Art

Recently, with the progress of the genetic engineering techniques, nucleic acids are widely purified from various cells, viruses and phages. For determining base sequences of DNAs carrying genetic information, it is an important technique to replicate single-stranded DNAs by culturing M13 phage with *E. coli*. Purification of phage DNAs is generally carried out in this technique. The widely adopted conventional method for purifying phage DNAs from the culture medium comprises the steps of removing *E. coli* cells from the culture medium by centrifugation, precipitating the phage by treatment with polyethyleneglycol, removing proteins by phenol extraction and concentrating DNA by ethanol precipitation.

However, with this conventional method, reagents which are toxic to human, such as phenol and chloroform must be used. Further, since centrifugation is necessary in each step, it is difficult to automatize the method. For automatizing the purification, a method which does not require centrifugation is desired.

SUMMARY OF THE INVENTION

Accordingly, the object of the present invention is to provide a process of purifying phage DNAs by which phage DNAs may be purified to high purity without centrifugation steps and without using reagents toxic to human.

The present inventors intensively studied to find that phage DNAs may be purified to high purity by removing *E. coli* cells by filtration through a membrane filter, decomposing and denaturing the phage proteins, and then removing the proteins by ultrafiltration, to complete the present invention.

That is, the present invention provides a method for purifying phage DNAs comprising, in the order mentioned, the steps of filtering a mixture containing microorganism cells and phage through a membrane filter to obtain a filtrate, so as to remove said microorganism cells; decomposing and denaturing phage proteins in said filtrate; and subjecting the resultant to ultrafiltration so as to remove impurities.

By the method of the present invention, phage DNAs may be purified to high purity with high yield from a culture medium without conducting a centrifugation step. According to the method of the present invention, since the centrifugation steps is not necessary, the method may be automatized. Further, with the method of the present invention, toxic reagents are not necessary and the time required for the purification is shortened.

BRIEF DESCRIPTION OF THE DRAWING

The drawing shows an agarose gel electrophoresis pattern of a phage DNA purified by the method of the present invention together with an electrophoresis pattern of the phage DNA purified by the conventional method and an electrophoresis pattern of a commercially available phage DNA.

BEST MODE FOR CARRYING OUT THE INVENTION

In the method of the present invention, a mixture containing microorganism cells and phage is treated. Representative example of such a mixture is a culture medium of a microorganism which is infected with a virus. The culture medium may be treated as it is by the method of the present invention. For example, a culture medium containing M13 phage and *E. coli* in 2 x TY medium or the like may be treated by the method of the present invention.

In the first step of the method of the present invention, the above-described mixture is filtered through a membrane filter so as to remove microorganism cells. The pore size of the membrane filter used here may preferably be 0.45 - 0.22 μm. The material constituting the membrane filter is not at all restricted.

In the second step, the phage proteins in the filtrate is decomposed and denatured. The decomposition and denaturation of the proteins may be carried out by treating the filtrate with a proteolytic enzyme such as Proteinase K. In this case, the concentration of the proteolytic enzyme may be appropriately selected. When a culture medium containing M13 phage and *E. coli* is treated, the concentration of the proteolytic enzyme may usually be 0.001 - 0.5 mg/ml. As described later, it is preferred to subject the filtrate to ultrafiltration before the treatment with a proteolytic enzyme and to add the proteolytic enzyme to the phage remained on the ultrafiltration membrane, although this ultrafiltration step is not required.

Other than the treatment with a proteolytic enzyme, the decomposition and denaturation of the

phage proteins may be carried out by treatment with an organic solvent, a surfactant or an alkali or by heat treatment or the like. More particularly, the step of decomposing and denaturing the proteins may be carried out by treating the protein with an organic solvent such as 30 - 100% methanol or ethanol. The separation of DNA and proteins may be attained by using a reagent which does not have a decomposing ability as strong as proteolytic enzymes or organic solvents, when a comparatively simple phage is to be treated. For example, the decomposition and denaturation of proteins may be attained by treatment with a surfactant. Preferred examples of the surfactant include anion surfactants such as sodium lauryl sulfate (SDS). In case of using SDS, the concentration thereof may preferably be 0.01 - 1% by weight. Since proteins are weak to heat and nucleic acids such as DNAs are relatively strong to heat, heat may be utilized for the protein-nucleic acid separation. In this case, the heat treatment may preferably be carried out at a temperature of 80 - 100°C for 5 - 20 minutes. The decomposition and denaturation of the proteins may also be carried out by alkali treatment. Preferred examples of the alkali include aqueous solution of alkaline metal hydroxides with a concentration of 0.1 - 1N.

The above-mentioned treatments may be carried out individually or in combination. By this step, proteins can be removed from the phage DNA.

The resulting solution is then ultrafiltered. The ultrafiltration membrane used herein preferably has a fractionation molecular weight of 20,000 to 1,000,000. If the molecular weight to be fractionated is smaller than this range, the efficiency of the removal of proteins is reduced. On the other hand, if the molecular weight is larger than this range, DNA may pass through the ultrafilter. The ultrafilter may be made of any material. For example, commercially available ultrafilters made of polysulfone may be employed. After the ultrafiltration, by washing the ultrafilter with an appropriate solution such as a buffer solution, the phage DNA may be recovered as a solution. To promote the yield of the phage DNA, it is preferred to wash the ultrafilter by passing the solution through the ultrafilter. The solution to be used for washing the ultrafilter is not restricted. Widely used buffer solutions such as TE-buffer (Tris-HCl buffer containing EDTA) may be employed as the solution for washing the ultrafilter. By this ultrafiltration step, the proteins and other impurities are removed from the solution obtained in the second step, so that phage DNA is purified.

The second step may be carried out immediately after the first step as described above. However, after the first step, the obtained filtrate may be subjected to ultrafiltration to remove low molecular components, thereby purifying the phage. In this case, the ultrafiltration may be carried out in the same manner as in the third step just described above. Further, if this ultrafiltration step is employed, the treatment for decomposing and denaturing the proteins in the second step may be carried out on the ultrafilter, and the ultrafiltration in the third step may be carried out on the same ultrafilter.

Example

The invention will now be described by way of an example thereof. The example is presented for illustration purpose only and should not be interpreted in any restrictive way.

E. coli JM109 was inoculated to 2 x TY medium, and then M13 phage was infected thereto. The medium was incubated at 37°C for 4 hours.

The resulting culture medium was filtered through a membrane filter having a pore size of 0.45 μm under a pressure with nitrogen gas.

The obtained filtrate was subjected to ultrafiltration through a ultrafilter having a fractionation molecular weight of 300,000 to remove low molecular weight components in the culture medium. To decompose the proteins of the phage, 5 μg/μl solution of proteinase K was placed on the ultrafilter, and the reaction was allowed to occur for 10 minutes.

The enzyme solution was removed under pressure, and the ultrafilter was washed with TE buffer by passing through the buffer under pressure through the ultrafilter. Two hundred microliters of TE buffer was placed on the ultrafilter and the ultrafilter was shaken. Thereafter, the TE buffer was recovered by using a pipette.

By this procedure, 2.5 μg of phage DNA was recovered from 2 ml of the culture medium.

The thus obtained phage DNA was subjected to agarose gel electrophoresis according to a conventional method. As controls, commercially available M13 phage DNA and the M13 phage DNA purified by the conventional purification method comprising polyethylene glycol precipitation and phenol extraction were also subjected to agarose gel electrophoresis.

The results are shown in the drawing. In the drawing, lane 1 shows an electrophoresis pattern of molecular weight markers consisting of *Hin*dIII digest of λ phage, lane 2 shows an electrophoresis pattern of commercially available M13 phage single-stranded DNA, lane 3 shows an electrophoresis pattern of M13 phage DNA purified by the conventional polyethylene glycol precipitation and phenol extraction, and lane 4 shows an electrophoresis pattern of M13 phage DNA purified by the method of the present invention.

As is apparent from the drawing, it was confirmed that the phage DNA was purified to high purity by the method of the present invention.

Although the present invention was described by way of preferred embodiment thereof, it is apparent for those skilled in the art that various modifications may be made without departing from the spirit and scope of the present invention.

Claims

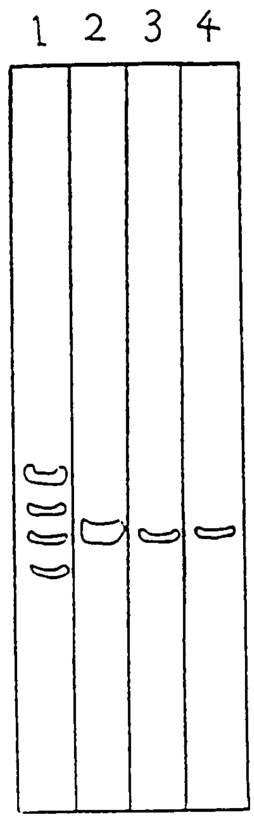
1. A method for purifying phage DNA comprising, in the order mentioned, the steps of:
   filtering a mixture containing microorganism cells and phage through a membrane filter to obtain a filtrate, so as to remove the said microorganism cells;
   decomposing and denaturing phage proteins in the said filtrate; and
   subjecting the resultant to ultrafiltration so as to remove impurities.

2. A method as claimed in Claim 1, characterised in that the pore size of the said membrane filter is 0.45 μm to 0.22 μm.

3. A method as claimed in Claim 1 or Claim 2 characterised in that the molecular weight to be fractionated by the said ultrafiltration is in the range 20,000 to 1,000,000.

4. A method as claimed in Claim 1, 2 or 3 characterised in that the step of decomposing and denaturing phage proteins is carried out by treating the said phage proteins with a proteolytic enzyme.

5. A method as claimed in Claim 1, 2, 3 or 4 characterised in that it further comprises the step of ultrafiltration of the said filtrate obtained in the first step from the membrane filter before the said step of decomposing and denaturing the phage proteins.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